

REGULATION OF HER2/neu ONCOGENE EXPRESSION BY SYNTHETIC
POLYAMIDES

The U.S. Government has certain rights in this invention pursuant to Grant
5 Nos. GM 26453, 27681 and AI 29182 awarded by the National Institutes of Health.

RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application
60/099,906, filed September 11, 1998, which is hereby incorporated by reference as if
10 fully set forth.

FIELD OF THE INVENTION

This invention is directed generally to methods and compositions for the
modulation, or regulation, of gene expression by the use of polyamides that bind
15 DNA. The methods and compositions result in inhibition, or down-regulation, of
gene expression or overexpression by interactions between the polyamides and the
minor groove of double-stranded DNA (dsDNA). The polyamides of these methods
and compositions bind predetermined target nucleic acid sequences located within the
promoter region of genes to be down-regulated or inhibited. Inhibition or down-
20 regulation of target oncogenes that are expressed, or overexpressed, at undesirable
levels is one application of the invention. In particular, the invention is directed to
reducing the expression or overexpression of target endogenous cellular oncogenes.

BACKGROUND OF THE INVENTION

25 The tyrosine kinase membrane growth factor receptor HER2/neu, also known
as p185^{HER2}, is encoded by a cellular oncogene of the same name that is
overexpressed and amplified in 20 to 30% of human breast cancers. See Baert, J.-L. et
al., *Int. J. Cancer* 70, 590-597 (1997); Benz, C., et al., *Oncogene* 15, 1513-1525
(1997); Chang, C.-H., et al., *Oncogene* 14, 1617-1622 (1997); Scott, G. K., et al., *J.*
30 *Biol. Chem.* 269, 19848-19858 (1994); Pasleau, F.; et al., *Oncogene* 8, 849-854
(1993); Tal, M. et al., *Molecular and Cellular Biology* 7, 2597-2601 (1987). Her-

2/neu is also overexpressed in other conditions, including human gynecologic adenocarcinomas, such as those of the ovary, endometrium, breast, fallopian tube and cervix (Cirisano, F.D., & Karlan, B.Y., *J. Soc. Gynecol. Investig.* 3 99-105 (1996)).

The neu oncogene gene product was originally described in chemically-induced (ethylnitrosourea) tumors in rodents. Subsequently, the human counterpart, c-erbB-2 or Her-2/neu, was found to be homologous to the EGF receptor, a 185 kDa transmembrane protein with protein tyrosine kinase activity. Overexpression of Her2/neu is also associated with the likelihood that tumors will metastasize, with a resulting poor prognosis for the patient. Mutation, amplification, and overexpression of the Her2/neu oncogene has been reported to be associated with breast tumor progression, early metastasis, and poor prognosis. Her2/neu gene amplification is directly correlated with lymph node metastasis. Additionally, in an animal model, activating mutations lead to rapid tumor progression. It is believed that the Her-2/neu protein likely plays a role in cell motility and hence in metastasis. Thus, inhibition of Her-2/neu gene expression by direct interference at the DNA level may be a potent therapeutic approach for metastatic disease.

Several transcription factors - such as ESX, AP-2, and the TATA binding protein ("TBP") - play an important role in the regulation of the expression of the gene for the HER2/neu growth factor receptor. See Baert, J.-L. et al., supra; Benz, C., et al., supra; Chang, C.-H., et al., supra; Bosher, J. M., et al., *Proc. Natl. Acad. Sci. USA* 92, 744-747 (1995). These transcription factors activate the expression of p185HER2 upon binding to sites within the HER2/neu promoter. The nucleotide sequence of the HER2/neu promoter and a schematic representation are shown in Figure 1.

TBP is a ubiquitous transcription factor that is involved in the activation of most protein-encoding genes. TBP is a DNA-binding protein that interacts with the minor groove of DNA. It is noted that, apart from ESX, AP-2, and TBP, there are other potential transcription factor binding sites within the HER2/neu promoter.

Considerable effort has been expended in the art to devise methods to interfere with gene expression in living cells in the hope that therapeutic strategies will come from these studies. These approaches include interference with the translation of

messenger RNA into protein by the introduction of antisense oligonucleotides into cells (natural or peptide nucleic acid based) or by ribozyme-mediated destruction of specific RNAs. Several approaches for direct inhibition of gene transcription have also been attempted; these include triple helix forming oligonucleotides, designed or
5 selected zinc finger peptides that recognize pre-determined sequences, and DNA-binding calicheamicin oligosaccharides.

For any therapeutic approach based on interference with gene expression, to be successful, several criteria must be met by the therapeutic agent: first, the agent must not possess any general cell toxicity; second, the agent must be cell-permeable and, in
10 the case of the DNA-binding agents, the compounds must transit to the nucleus and bind their target sequence with high affinity and specificity in the context of cellular chromatin; and, third, binding of the agent to its DNA target sequence must interfere with gene transcription. Each of the potential approaches listed above has its own peculiar limitations. For example, while triple helix-forming oligonucleotides have
15 the potential for sequence selectivity and can effectively inhibit transcription *in vitro*, these molecules suffer from poor cell permeability and permeabilized cells need to be used for effective gene inhibition. Similarly, zinc finger peptides must be introduced via a gene therapy approach with an appropriate viral, or non-viral, expression vector since these peptides cannot directly enter cells. In contrast, the calicheamicin
20 oligosaccharides are sufficiently hydrophobic to pass through cell membranes, but these molecules possess severely limited sequence specificity (4 bp) and bind DNA with very low affinities (100 μ M or higher required for inhibition of protein-DNA interactions). Thus, new classes of cell-permeable molecules that possess higher degrees of DNA sequence specificity and affinity are needed for any human gene
25 therapeutic approach to be feasible.

Another approach utilizes cell-permeable small molecules that target particular DNA sequences. These molecules would be useful for the regulation of gene expression. The design of small synthetic DNA-binding ligands that recognize specific sequences in the DNA double helix has been a long standing goal of
30 chemistry. Oligodeoxynucleotides that recognize the major groove of double-helical DNA via triple-helix formation bind to a broad range of sequences with high affinity

and specificity. Although oligonucleotides and their analogs have been shown to interfere with gene expression, the triple helix approach is limited to purine tracks and suffers from poor cellular uptake.

Other small molecules have also been of interest as DNA-binding ligands. Wade, et al., reported the design of peptides that bind in the minor groove of DNA at 5'-(A,T)G(A,T)C(A,T)-3' sequences by a dimeric side-by-side motif (*J. Am. Chem. Soc.* **114**, 8783-8794 (1992)). Mrksich, et al. reported describes antiparallel side-by-side motif for sequence specific-recognition in the minor groove of DNA by the designed peptide 1-methylimidazole-2-carboxamidenetropsin (*Proc. Natl. Acad. Sci. USA* **89**, 7586-7590 (1992)). Pelton, J.G. & Wemmer, D.E. reported the structural characterization of a 2-1 distamycin A-d(CGCAAATTTGGC) complex by two-dimensional NMR (*Proc. Natl. Acad. Sci. USA* **86**, 5723-5727 (1989)).

Dervan and colleagues have shown that synthetic pyrrole-imidazole polyamides bind DNA with excellent specificity and very high affinities, even exceeding the affinities of many sequence-specific transcription factors (Trauger, et al., *Nature* **382**, 559-561 (1996)). They further describe the recognition of DNA by designed ligands at subnanomolar concentrations. DNA recognition depends on side-by-side amino acid pairing of imidazole-pyrrole or pyrrole-pyrrole pairs in the minor groove. White, S., et al., (1996) reported the effects of the A•T/T•A degeneracy of pyrrole-imidazole polyamide recognition in the minor groove of DNA (*Biochemistry* **35**, 6147-6152 (1996)). White, et al. (1997) reported pairing rules for recognition in the minor groove of DNA by pyrrole-imidazole polyamides (*Chem. & Biol.* **4**, 569-578 (1997)), and demonstrated the 5'-3' N-C orientation preference for polyamide binding in the minor groove. Thus polyamide molecules thus have the potential to act as inhibitors of protein-DNA interactions in the minor groove.

The development of pairing rules for minor groove binding polyamides derived from N-methylpyrrole (Py) and N-methylimidazole (Im) amino acids provides another means to confer sequence specificity. An Im/Py pair distinguishes G•C from C•G, and both of these from A•T or T•A base pairs, while Py/Im targets a C-G basepair. A Py/Py pair specifies A•T from G•C but does not distinguish A•T from

T•A. The generality of this approach to the rational design of sequence-specific DNA ligands is supported by direct NMR structural studies (Geierstanger, et al., *Science* 266,646-650 (1994)) and the recent success in synthesis of an eight ring hairpin polyamide which targets a six base pair sequence with an apparent dissociation constant of 0.03 nM (Trauger et al., above). Moreover, two eight-ring pyrrole-imidazole polyamides differing in sequence by a single amino acid residue bind specifically to respective six base-pair target sites that differ in sequence by a single base pair. The replacement of a single nitrogen atom with a C-H can regulate specificity and affinity by two orders of magnitude.

Since a six base-pair sequence would be highly redundant in the human genome (occurring at random once every 4 kilobases, or 500,000 times, in the human genome), polyamides have been synthesized to recognize much longer sequences. For example, a twelve-ring double hairpin polyamide has been designed to target a 12 bp site and binding is again observed with nanomolar affinity. Such a sequence would be predicted to occur at random only once every 16 million base pairs, or only 125 times, in the human genome. Such molecules thus have the potential to act as specific inhibitors of gene transcription *in vivo* and as human therapeutic agents if the conditions outlined above can be met.

SUMMARY OF THE INVENTION

The present invention relates to and includes methods and compositions for the modulation, or regulation, of gene expression or overexpression by reducing the transcription of genes. Preferably, the transcription of specific individual target genes is reduced. Such reductions result from the application of polyamides that bind or interact with the minor groove of double-stranded (dsDNA) within the promoter regions of target genes. Preferably, the binding or interaction is with a predetermined target nucleic acid sequence within the promoter regions to inhibit or down-regulate transcription.

The present invention reduces gene expression and overexpression by use of sequence-specific DNA-binding small molecules that are cell-permeable and capable of inhibiting gene transcription. Appropriate application of such molecules may

inhibit overexpression of endogenous oncogenes to provide a fundamentally new therapeutic strategy for the treatment of various diseases, including cancer. The small molecules of the invention are polyamides that bind to or interact with nucleic acid sequences within the promoter region of target genes. Preferably, these sequences are recognized, or proximal to those that are recognized, by one or more transcription factors.

Preferably, the polyamides bind to the minor groove of double-stranded DNA in a promoter region that controls the transcription and expression of a gene. Preferably the transcription of the gene is inhibited by modulating the binding of a protein transcription factor to dsDNA. In preferred embodiments, the transcription factors are ESX, ETS, and TBP.

Previous studies directed toward inhibition of the transcriptional activity of the HIV-1 promoter have demonstrated that polyamides can block binding of TBP as well as an *Ets* family transcription factor (see PCT published application PCT/US98/02444, now WO 98/35702, the teachings of which are incorporated by reference as if fully set forth). In principle, both classes of transcription factors can be inhibited by polyamides that contact or bind the minor groove of dsDNA. Without being bound by theory, DNA complexation of proteins contacting the minor groove may be inhibited by direct steric hindrance, repulsion or exclusion. Alternatively, inhibition may be by allosteric effects. In comparison, and again without being bound by theory, DNA complexation of proteins contacting the major groove may be suppressed by a polyamide-induced change in DNA conformation. Of course, inhibition can also be achieved by other means, for example, by conjugating a DNA cleavage agent to a polyamide targeted to a desired site, or by chemically modifying DNA.

In one preferred aspect of the invention, the expression or overexpression of oncogenes is targeted. Preferably, the oncogenes are endogenous cellular oncogenes involved in cancer, particularly human breast cancer. One oncogene target of the invention is the HER-2/neu gene, which may be down-regulated or inhibited by the use of polyamides that bind to target sequences within the HER-2/neu promoter region. Preferably, these sequences are, or are proximal to, transcription factor

binding sites within the HER2/neu promoter. Interactions or binding between the polyamide and the target sequence can inhibit the transcription of the HER2/neu gene. The degree of inhibition of HER2/neu expression may be extensive and includes the inhibition of HER2/neu overexpression. The invention further encompasses
5 application of polyamides for the treatment of various tumors or cancers, including breast cancer.

Suitable polyamides have a binding affinity at the dsDNA target sequence of at least 10^9 M^{-1} and a selectivity of at least about two. Selectivity is defined as the ratio of the binding affinity for the identified dsDNA target sequence to the binding
10 affinity for a single base-pair mismatch dsDNA sequence. In preferred embodiments, selectivity against at least 90% of single base mismatch sequences is greater than about 10.

The present invention also provides compositions comprising a pharmaceutically acceptable excipient and a transcription-inhibiting amount of at least
15 one polyamide of the invention. Each polyamide contains at least four complementary pairs of aromatic carboxamide residues, which pairs are selected to correspond to an identified nucleotide sequence of a dsDNA target. The polyamides additionally comprise at least two aliphatic amino acid residues chosen from the group consisting of glycine, β -alanine, γ -aminobutyric acid, R-2,4-diaminobutyric acid, and
20 5-aminovaleric acid, and at least one terminal alkylamino residue, the polyamide having a binding affinity at the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified target dsDNA sequence to the binding affinity for a single base-pair mismatch dsDNA sequence.

25 The invention further provides methods and compositions suitable for treating a subject having a condition associated with abnormal expression of a cellular oncogene. The subject is preferably a human patient, particularly one afflicted with breast cancer or other diseases or conditions associated with aberrant Her-2/neu oncogene expression. The methods comprise administration of polyamides or
30 compositions as described above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the HER2/neu promoter, showing the nucleotide sequence in A, including binding sites of Ets, AP-2, and TBP ("TATA") transcription factors and the "CCAAT box", and in B, a schematic diagram, not to scale.

5

Figure 2A is a graphical representation of the results of a DNase I footprint titration of polyamide **HER2-1** (left) and the mismatch polyamide ImPy- β -PyIm- γ -PyPyPyPyPy- β -Dp (right) and 2B, the schematic structures and association constants of the polyamides, where the polyamides are represented by closed circles for imidazole rings, open circles for pyrrole rings, curved lines for γ -aminobutyric acid, diamonds for β -alanine, and a half circle with a positive charge for dimethylaminopropylamide.

10

Figure 3 compares the sequence of the HER2/neu promoter and polyamide structures and binding sites; the binding site for the TATA binding protein (TBP) is indicated along with the structures of the polyamides HER2-A, HER2-1, 70, and the mismatch polyamide 86.

15

Figure 4 is a graphical representation of the results of experiments showing the effects of polyamides Her2-1 (A) and 70 on TBP binding.

20

Figure 5 is a graphical representation of the results of experiments showing the effects of the polyamide HER2-1 on HER2/neu transcription *in vitro* in a cell free system.

25

Figure 6 is a graphical representation of the results of experiments showing the effects of the polyamides HER2-1 and 70 on HER2/neu mRNA production in the human breast cancer cell line SK-BR-2.

Figure 7 shows binding models for polyamides **1a** - **1c**. The double-stranded DNA sequence encompassing the Her-2/neu TATA element and flanking sequence is shown, with the TATA element boxed. Polyamides are represented schematically between the two DNA strands at their respective binding sites. The closed and open circles represent Im and Py rings, respectively, the diamonds represent β -alanine (β) and the curved lines represent γ -aminobutyric acid (γ). The charged chiral turn ((R)-2,4-diaminobutyric acid) is represented with a plus sign as is the terminal dimethylaminopropylamide. Equilibrium association constants for each polyamide were determined by quantitative DNase I footprinting and are indicated.

Figure 8 shows polyamide inhibition of TBP binding to a 35 bp oligonucleotide corresponding to the Her-2/neu TATA element and flanking sequence. Results of the EMSA were determined by phosphorimage analysis and are plotted as the fraction of bound complex remaining as a function of polyamide concentration for polyamides Her2-1 (shown as polyamide **1**), **1a**, **1b**, **1c**, and a mismatch polyamide **4** (ImPy- β -ImPy- γ -ImPy- β -ImPy- β -Dp).

DETAILED DESCRIPTION

The present invention is directed to methods and compositions for modulating or regulating gene expression or overexpression by reducing gene transcription. The methods and compositions are preferably directed toward the inhibition of oncogene transcription, especially of oncogenes involved in cancer, particularly human cancer, and especially breast cancer.

The reductions in gene transcription result from binding or other interactions between polyamides and the minor groove of dsDNA within the promoter regions of target genes. Preferably, the polyamides bind or interact with specific, or predetermined, target nucleic acid sequences within the promoter regions to inhibit or down-regulate transcription. Predetermined sequences refer to those sequences for which binding by a polyamide would be desirable. The actual sequences may be known or merely identified by location or other characteristics. Preferably, the sequences are

recognized, or proximal to those that are recognized, by one or more transcription factors.

The polyamides are preferably cell-permeable and capable of inhibiting gene transcription *in vivo*, *in vitro*, or in cell free systems. Appropriate application of such polyamide molecules may be used to inhibit expression or overexpression of endogenous oncogenes as a treatment of various diseases, including cancer.

In preferred embodiments, the polyamides bind to the minor groove of double stranded DNA in a promoter region that controls the transcription and expression of a target gene. Preferred target genes are endogenous oncogenes involved in cancer formation or progression. Preferably the transcription of the gene is inhibited by modulating the binding of a protein, such a transcription factor, to the same promoter region with which the polyamide binds or interacts. In especially preferred embodiments, the transcription factors are one or more of the following: ESX, ETS and TBP.

Inhibition of transcriptional activity at the HIV-1 promoter have demonstrated that polyamides can block binding of TBP as well as an *Ets* family transcription factor (see WO 98/35702, which includes a discussion of polyamide synthesis). The present invention includes the use of polyamides that inhibit or modulate the activity of both TBP and *Ets* transcription factors. The invention may affect transcription factor activity by use of one or more polyamides that contact or bind the minor groove of dsDNA. Such contact or binding may inhibit formation of DNA-transcription factor complexes in the minor groove by direct steric hindrance, repulsion, allosteric effects, or other mechanisms. This is possibly in contrast to major groove DNA binding proteins, such as TBP, which may be inhibited by a polyamide-induced change in DNA conformation.

In a preferred aspect of the invention, the expression or overexpression of oncogenes, especially endogenous cellular oncogenes, is targeted. Preferably, the oncogenes are those implicated in human breast cancer, and their expression or overexpression is inhibited by polyamides that contact or bind the minor groove in the region of the oncogene promoter. Preferably, the contacted or bound portions of the promoter region are, or are proximal to, transcription factor binding sites. The degree

of inhibition is preferably large and more preferably enough to inhibit even overexpression of the oncogene, in which the copy number of the gene may have increased.

One oncogene target of the invention is the HER-2/neu gene, which may be
5 down-regulated or inhibited by the use of polyamides that bind to target sequences within the HER-2/neu promoter region. Preferably, these sequences are, or are proximal to, transcription factor binding sites within the HER2/neu promoter. These transcription factors include TBP, ESX and AP-2. Interactions or binding between the polyamide and the target sequence result in inhibition of the HER2/neu gene
10 transcription.

In a preferred embodiment, a polyamide was designed to bind immediately downstream of the TATA element found in the human Her-2/neu breast cancer oncogene promoter. This polyamide, Her2-1, of composition ImPy- β -PyIm- γ -PyPy- β -PyPy- β -Dp, binds the sequence 5'-AGAATGA-3' (where the 5' A of this sequence
15 is the 3' A of the TATA element) with an apparent dissociation constant of 200 pM. Her2-1 is an effective inhibitor of TBP binding and transcription.

The present invention includes compositions comprising a pharmaceutically acceptable excipient and a transcription-inhibiting amount of at least one polyamide for the inhibition of gene expression or overexpression. These compositions may also
20 be used for the treatment of various tumors or cancers, including breast cancer. The invention further provides methods of administering such compositions to result in inhibition of gene expression or overexpression. The methods and compositions are preferably suited for treating a subject having a condition associated with abnormal expression of a cellular oncogene. The subject is preferably a human patient,
25 particularly one afflicted with cancer, especially breast cancer.

Polyamides of the invention

The polyamides used in the present invention comprise N-methylimidazole and N-methylpyrrole carboxamides. These polyamides generally have a crescent-
30 shaped structure that permits interaction and complexation the minor groove of double-stranded DNA. NMR studies have confirmed that these compounds can bind

to DNA in a 2:1 ratio by a motif in which two polyamide ligands are arranged in an antiparallel way, side-by-side to each other (Pelton, J., et al., *Proc. Natl. Acad. Sci. USA* **86**, 5723-5727 (1986); Mrksich, M., et al., *Proc. Natl. Acad. Sci. USA* **89**, 7586-7590 (1992); Wade, W. S., et al., *J. Am. Chem. Soc.* **114**, 8783 (1992)).

5 One means to increase the binding affinity of two polyamides is to covalently linked them with a turn-unit such as γ -aminobutyric acid (see Mrksich, M., et al., *J. Am. Chem. Soc.* **116**, 7983 (1994)). Such polyamides are called "hairpin polyamides", as they adopt a hairpin-like conformation in the DNA complex. The sequence of the imidazole and the pyrrole carboxamides in the polyamide determines the DNA
10 sequence specificity of the ligand, according to the scheme of carboxamide pairs that recognize nucleotide pairs described above. In some cases it has been useful to replace one or several pyrrole carboxamide units with β -alanine moieties in order to adjust the curvature of the polyamide to that of the DNA. It has recently been shown that polyamides comprising N-methylimidazole and N-methylpyrrole carboxamides can
15 inhibit gene expression in eukaryotic cells (Gottesfeld, J.M., et al. *Nature* **387**, 203-205 (1997)).

It has been found that polyamides containing a new aromatic amino acid, 3-hydroxy-N-methylpyrrole (Hp), paired opposite Py, have the ability to discriminate A•T nucleotide pairs from T•A nucleotide pairs in DNA sequences. These pairing rules are supported by
20 direct NMR structural studies (Geierstanger, et al. (1994) Design of a G• C-specific DNA minor groove-binding peptide. *Science* **266**, 646-650) and by X-ray crystallography (Kielkopf, et al. (1998) Structural basis for GC recognition in the DNA minor groove. *Nature Struct. Biol.* **5**, 104-109; Kielkopf, et al. (1998) A structural basis for recognition of A.T and T.A base pairs in the minor groove of B-DNA. *Science* **282**, 111-115). The Py-Im
25 polyamides have affinities and specificities comparable to those of natural eukaryotic DNA-binding transcription factors: for example, an eight ring hairpin polyamide, which targets a six base pair sequence, binds with an apparent association constant of $3 \times 10^{10} \text{ M}^{-1}$ (Trauger, et al. (1996) Subnanomolar sequence-specific recognition in the minor groove of DNA by designed ligands. *Nature* **382**, 559-561). Moreover, two eight-ring pyrrole-imidazole

polyamides differing in sequence by a single amino acid bind specifically to respective six base-pair target sites which differ in sequence by a single base pair.

The replacement of a single hydrogen atom on the pyrrole with a hydroxyl group in a Hp/Py pairing affects the affinity and specificity of a polyamide by an order of magnitude. The replacement of a single nitrogen atom with a C-H can regulate specificity and affinity by two orders of magnitude. By using Hp together with Py and Im in four pairs of aromatic amino acid residue combinations (Im/Py, Py/Im, Hp/Py, and Py/Hp), polyamides can selectively distinguish all four Watson-Crick base pairs in the minor groove of double stranded DNA. White, et al., *Nature* 391, 468-471 (1998).

The invention encompasses the use of improved polyamides for binding to the minor groove of DNA in methods and compositions for reducing gene expression or overexpression. The preparation and use of polyamides for binding in the minor groove of DNA are described in the art. Included in the invention is an improvement of the existing technology which utilizes 3-hydroxy-N-methylpyrrole to provide carboxamide binding pairs for DNA binding polyamides. The improvement relates to the inclusion of a binding pair of Hp/Py carboxamides in the polyamide to bind to a T•A base pair in the minor groove of DNA or Py/Hp carboxamide binding pair in the polyamide to bind to an A•T base pair in the minor groove of DNA. The polyamides used in the invention have at least four carboxamide binding pairs that will distinguish A•T, T•A, C•G and G•C base pairs in the minor groove. The polyamides may also have γ -aminobutyric acid or another turn unit to form a hairpin-loop with a member of each carboxamide pairing on each side of it.

The invention also includes polyamides containing a β -alanine substituted for a Py residue that would ordinarily be used in a carboxamide binding pair to match a particular nucleotide pair. The β -alanine is referred to in formulas of this invention as β . The β -alanine becomes a member of a carboxamide binding pair, and serves to optimize hydrogen bonding of neighboring amino acid moieties to nucleotide base pairs. The invention further includes the substitution of a β • β binding pair for a non-

Hp containing binding pair. Thus, binding pairs in addition to the Hp/Py and Py/Py are Py/Py, Im/Py, Py/Im, Im/ β , β /Im, Py/ β , β /Py, and β / β .

In general, the polyamides of the invention are suitable for inhibiting the transcription of a gene, preferably an oncogene. The polyamides consist of at least
5 four complementary pairs of aromatic carboxamide residues, which pairs are selected to correspond to the nucleotide sequence of a dsDNA target. These polyamides contain at least two aliphatic amino acid residues chosen from the group consisting of glycine, β -alanine, γ -aminobutyric acid and 5-aminovaleric acid, and at least one terminal alkylamino residue. The complementary pairs of aromatic carboxamide
10 residues selected to correspond to the nucleotide sequence of an identified dsDNA target are chosen from the group consisting of Im/Py to correspond to the nucleotide pair G/C, Py/Im to correspond to the nucleotide pair C/G, Py/Py to correspond to the nucleotide pair A/T, Py/Py to correspond to the nucleotide pair T/A, Hp/Py to correspond to the nucleotide pair T/A, and Py/Py to correspond to the nucleotide pair
15 A/T, where Im is N-methyl imidazole, Py is N-methyl pyrrole and Hp is 3-hydroxy N-methyl pyrrole. Application of the above principles permits the design of specific polyamides that bind or interact with specific target nucleic acid sequences for use in reducing gene expression or overexpression.

Preferred polyamides contain at least one β -alanine as an aliphatic amino acid
20 residue. In preferred embodiments the terminal alkylamino residue is a N,N-dimethylaminopropyl residue. Suitable polyamides containing at least two β -alanine residues aligned to form complementary paired residues corresponding to a nucleotide pair chosen from the group A/T and T/A. Alternatively, corresponding pairs can be formed between aliphatic amino acids and aromatic carboxamides, such as Im/ β ,
25 β /Im, Py/ β and β /Py. In preferred polyamides, a hairpin molecule is formed by inclusion of an aliphatic amino acid residue such as γ -aminobutyric acid. Additionally, in some polyamides of the invention, at least one Py of a carboxamide pair is replaced by a β -alanine.

Suitable polyamides have a binding affinity at the dsDNA target sequence of
30 at least 10^9 M^{-1} and a selectivity of at least about two. Selectivity is defined as the ratio of the binding affinity for the identified dsDNA target sequence to the binding

affinity for a single base-pair mismatch dsDNA sequence. In preferred embodiments, selectivity against at least 90% of single base mismatch sequences is greater than about 10.

Each polyamide used in the compositions of the invention preferably contains
5 at least four complementary pairs of aromatic carboxamide residues, which pairs are selected to correspond to an identified nucleotide sequence of a dsDNA target. The polyamides also preferably contain at least two aliphatic amino acid residues chosen from the group consisting of glycine, β -alanine, γ -aminobutyric acid, R-2,4-diaminobutyric acid, and 5-aminovaleric acid, and at least one terminal alkylamino
10 residue. The polyamides also preferably have a binding affinity at the target dsDNA sequence of at least 10^9 M^{-1} and a selectivity of at least about two, selectivity being defined as the ratio of the binding affinity for the identified target dsDNA sequence to the binding affinity for a single base-pair mismatch dsDNA sequence.

Polyamide Her2-1 was designed to bind to the DNA sequence 5'-AGAATG-
15 3', which is immediately adjacent to the TATA box of the HER2/neu promoter. DNase 1 footprint analysis confirms that the polyamide binds to the desired sequence with a dissociation constant (K_d) of about 0.2 nM. Polyamide 70 also binds adjacent to, and partially overlaps, the HER2/neu TATA box. These polyamides targeted to the DNA sequences flanking or overlapping the Her-2/neu TATA element were
20 synthesized by solid phase methods.

Polyamide Her2-A, of sequence composition ImIm- β -PyIm- γ -PyPy- β -PyPy- β -Dp (where Im represents imidazole, Py represents pyrrole, γ represents γ -aminobutyric acid, β represents β -alanine, and Dp represents dimethylaminopropylamide), binds the sequence 5'-AGGAAGT-3' at the 5' boundary
25 of the Her-2/neu TATA element while polyamide Her2-1, of sequence composition ImPy- β -PyIm- γ -PyPy- β -PyPy- β -Dp, binds the sequence 5'-AGAATGA-3' at the 3' boundary of the TATA element (see Figure 1).

A mismatch polyamide of sequence composition ImIm- β -ImIm- γ -PyPy- β -PyPy- β -Dp (termed HIV-1) was also used in these studies. Polyamide 70, of
30 sequence composition ImPyPyPy- γ -PyPyPyPy- β -Dp, binds the sequence 5'-AGTATA-3' overlapping the TATA box, while polyamide 86, of sequence

composition ImPyImPy- γ -PyPyPyPy- β -Dp, is a mismatch polyamide, with a single atom substitution from polyamide 70.

Figure 1 shows the sequence of the Her-2/neu promoter region and the binding sites of several transcription factors. The hairpin polyamide ImPy- β -PyIm- γ -PyPy- β -PyPy- β -Dp (Her2-1) was synthesized to bind immediately downstream of the TBP binding site (Figure 2B, left). Polyamides 70 and Her2-A bind to sites overlapping the ESX/Ets site upstream from the TATA element (see Fig. 3) and have been found to inhibit the DNA binding activity of a recombinant polypeptide containing the DNA binding domain of Ets-1. Additional polyamides will be directed to the binding of ESX, the actual Ets family member that is believed to bind the Ets site adjacent to the TATA element (Chang, C.H., *et al.* (1997) ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene* 14, 1617-1622).

Quantitative DNase I footprinting experiments (Figure 2A) conducted on a ³²P-end-labeled restriction fragment isolated from a HER2/neu gene-containing plasmid (Ebbinghaus, *et al.* "Triplex formation inhibits HER-2/neu transcription in vitro." *J. Clin Invest.* 92:2433-2439 (1993)) revealed that Her2-1 binds its target sequence with an equilibrium association constant of about $5 \times 10^9 \text{ M}^{-1}$. The mismatch polyamide ImPy- β -PyIm- γ -PyPyPyPyPy- β -Dp (polyamide 86) bound the same sequence with an equilibrium association constant of $2 \times 10^8 \text{ M}^{-1}$ (Figure 2B, right). The footprinting experiments indicated that this polyamide also binds the 5'-AGGAAGT-3' single-base pair mismatch sequence proximal to the ESX binding site with comparable affinity.

The TATA box region and binding models for each of these polyamides are shown in Figure 3. In this figure, polyamides are represented schematically between the two DNA strands at their respective binding sites. Shaded and unshaded circles represent imidazole (Im) and pyrrole (Py) rings, respectively; curved lines represent γ -aminobutyric acid (γ); diamonds represent β -alanine (β); and Dp represents dimethylaminopropylamide. The apparent binding affinities for each of these polyamides was determined by quantitative DNase I footprint titrations. Polyamide Her2-A binds its match site with a K_a of $<10^8 \text{ M}^{-1}$ while polyamide Her2-1 binds with a K_a of $5 \times 10^9 \text{ M}^{-1}$. Given the higher affinity of Her2-1 for its target site, most of the

examples described below utilized this compound. The binding constant for polyamide 70 has been reported previously (polyamide 2 in Trauger *et al.*, *Nature* **382**, 559-561, 1996) and corresponds to $3.5 \times 10^9 \text{ M}^{-1}$. The mismatch polyamides bind with greatly reduced affinity.

5 Additional polyamides that bind adjacent to the TATA element have been synthesized (see Figure 7). These compounds are variants of polyamide Her2-1, described above, and include one with a charged chiral turn ((R)-2,4-diaminobutyric acid = D; ImPy- β -PyIm-D-PyPy- β -PyPy- β -Dp, **1a**) in place of the neutral, achiral γ -aminobutyric acid linker used in polyamide Her2-1. As a consequence, this polyamide is doubly charged, as are two
10 additional variants of polyamide Her2-1: one with a ring/beta pairing (ImPy- β -PyIm-D-PyPyPyPyPy- β -Dp, **1b**) and one with an additional two pyrrole amino acid units, which extend into the TATA box (ImPy- β -PyIm-D-PyPy- β -PyPy- β -PyPy- β -Dp, **1c**). Each of these compounds bind their target sequences with subnanomolar affinities (given in Fig. 7) and are effective inhibitors of TBP DNA binding activity, with polyamide **1c** being the most potent
15 inhibitor (Fig. 8). Based on these findings, we predict that these polyamides will be potent inhibitors of basal, TATA-dependent transcription.

20 Pharmaceutical and therapeutic compositions

The polyamides of the invention, as well as the pharmaceutically acceptable salts thereof, may be formulated into pharmaceutical or therapeutic compositions, formulations or preparations. Pharmaceutically acceptable salts of the polyamide compounds of the invention are formed where appropriate with strong or moderately
25 strong, non-toxic, organic or inorganic acids or bases by methods known in the art. Exemplary of the salts that are included in this invention are maleate, fumarate, lactate, oxalate, methanesulfonate, ethanesulfonate, benzenesulfonate, tartrate, citrate, hydrochloride, hydrobromide, sulfate, phosphate and nitrate salts.

As stated above, the polyamide compounds of the invention possess the ability
30 to inhibit gene expression or overexpression, properties that are exploited in the

treatment of any of a number of diseases or conditions, most notably cancer and especially breast cancer. A composition of this invention may be active *per se*, or may act as a "pro-drug" that is converted *in vivo* to an active form.

5 The compounds of the invention, as well as the pharmaceutically acceptable salts thereof, may be incorporated into convenient dosage forms, such as capsules, impregnated wafers, tablets or injectable preparations. Solid or liquid pharmaceutically acceptable carriers may be employed. Pharmaceutical compositions designed for timed release may also be formulated.

10 Preferably, the compounds of the invention are administered systemically, *e.g.*, by injection. When used, injection may be by any known route, preferably intravenous, subcutaneous, intramuscular, intracranial or intraperitoneal. Injectables can be prepared in conventional forms, either as solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

15 Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline, water, dextrose, glycerol and the like. Similarly, the carrier or diluent may include any prolonged release material, such as glyceryl monostearate or glyceryl distearate, alone or with a wax. When a liquid carrier is used, the preparation may be in the form of a syrup, elixir, emulsion, 20 soft gelatin capsule, liquid containing capsule, sterile injectable liquid (*e.g.*, a solution), such as an ampoule, or an aqueous or nonaqueous liquid suspension. A summary of such pharmaceutical compositions may be found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton Pennsylvania (Gennaro 18th ed. 1990).

25 The pharmaceutical preparations are made following conventional techniques of pharmaceutical chemistry involving such steps as mixing, granulating and compressing, when necessary for tablet forms, or mixing, filling and dissolving the ingredients, as appropriate, to give the desired products for oral or parenteral, including topical, transdermal, intravaginal, intranasal, intrabronchial, intracranial, 30 intraocular, intraaural and rectal administration. The pharmaceutical compositions

may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and so forth.

Although the preferred routes of administration are systemic, the pharmaceutical composition may be administered topically or transdermally, e.g., as an ointment, cream or gel; orally; rectally; *e.g.*, as a suppository, parenterally, by injection or continuously by infusion; intravaginally; intranasally; intrabronchially; intracranially intra-aurally; or intraocularly.

For topical application, the compounds and compositions of the invention may be incorporated into topically applied vehicles such as a salve or ointment. The carrier for the active ingredient may be either in sprayable or nonsprayable form. Non-sprayable forms can be semi-solid or solid forms comprising a carrier indigenous to topical application and having a dynamic viscosity preferably greater than that of water. Suitable formulations include, but are not limited to, solution, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like. If desired, these may be sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers, or salts for influencing osmotic pressure and the like. Preferred vehicles for non-sprayable topical preparations include ointment bases, e.g., polyethylene glycol-1000 (PEG-1000); conventional creams such as HEB cream; gels; as well as petroleum jelly and the like.

Also suitable for topical application are sprayable aerosol preparations wherein the compound, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, perfumes, and/or antioxidants in addition to the compounds of the invention.

For the preferred topical applications, especially for humans, it is preferred to administer an effective amount of the compound to a target area, *e.g.*, skin surface, mucous membrane, eyes, *etc.* This amount will generally range from about 0.001 mg to about 1 g per application, depending upon the area to be treated, the severity of the symptoms or disease, and the nature of the topical vehicle employed.

The compositions of the invention may be administered in combination with one or more additional compounds that are used to treat the disease or condition. For treating cancer, the polyamides and derivatives are given in combination with anti-tumor agents, such as mitotic inhibitors, *e.g.*, vinblastine; alkylating agents, *e.g.*, cyclophosphamide; folate inhibitors, *e.g.*, methotrexate, pritrexim or trimetrexate; antimetabolites, *e.g.*, 5-fluorouracil and cytosine arabinoside; intercalating antibiotics, *e.g.*, adriamycin and bleomycin; enzymes or enzyme inhibitors, *e.g.*, asparaginase; topoisomerase inhibitors, *e.g.*, etoposide; or biological response modifiers, *e.g.*, interferon. In fact, pharmaceutical compositions comprising any known cancer therapeutic in combination with the polyamine analogues and derivatives disclosed herein are within the scope of this invention.

Typical single dosages of the compounds of this invention are between about 1 ng and about 10 g/kg body weight. The dose is preferably between about 0.01mg and about 1g/kg body wt. and, most preferably, between about 0.1mg and about 100mg/kg body wt. For topical administration, dosages in the range of about 0.01-20% concentration of the compound, preferably 1-5%, are suggested. A total daily dosage in the range of about 1-500 mg is preferred for oral administration. The foregoing ranges are, however, suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are expected and may be routinely made by those skilled in the art.

Effective amounts or doses of the compound for treating a disease or condition can be determined using recognized *in vitro* systems or *in vivo* animal models for the particular disease or condition. In the case of cancer, many art-recognized models are known and are representative of a broad spectrum of human tumors. The compounds and compositions of the invention may be tested for inhibition of tumor cell growth in culture using standard assays with any of a multitude of tumor cell lines of human or nonhuman animal origin. Many of these approaches, including animal models, are described in detail in Geran, R.I. *et al.*, "Protocols for Screening Chemical Agents and

Natural Products Against Animal Tumors and Other Biological Systems (Third Edition)", *Canc. Chemother. Reports*, Part 3, 3:1-112.

Administration methods

5 As noted above, the treatment methods of the invention are directed to the administration of polyamide containing compositions. The polyamide containing preparations of the invention may be administered systemically or locally and may be used alone or as components of mixtures. The route of administration may be topical, intravenous, oral, or by use of an implant. For example polyamides may be
10 administered by means including, but not limited to, topical preparations, intravenous injection or infusion, oral intake, or local administration in the form of intradermal injection or an implant. Additional routes of administration are subcutaneous, intramuscular, or intraperitoneal injections of the polyamides in conventional or convenient forms. Liposomal or lipophilic formulations may also be used when
15 desired. For topical administration, the polyamides may be in standard topical formulations and compositions including lotions, suspensions or pastes. Oral administration of suitable formulations may also be appropriate in those instances where the polyamides may be readily administered to the target cells or tissues via this route.

20 The dose of polyamides may be optimized by the skilled artisan depending on factors such as, but not limited to, the polyamides chosen, the physical delivery system in which it is carried, the individual subject, and the judgment of the skilled practitioner.

25 For example, the form of administration, such as when coupled to a target-specific ligand, such as an antibody or an immunologically active fragment thereof, is one factor considered by a skilled artisan.

 Generally the dosages will depend on the specificity of the preparation, so that smaller or larger doses of polyamides may be administered as needed. For compositions
30 which are highly specific to the target tissues and cells, dosages in the range of 0.005-1

mg/kg of body weight are suggested. For compositions which are less specific to the target, larger dosages, up to 1-10 mg/kg, may be desirable. The foregoing ranges are merely suggestive in that the number of variables with regard to an individual treatment regime is large and considerable deviation from these values may be expected. The skilled artisan is free to vary the foregoing concentrations so that the uptake and stimulation/restoration parameters are consistent with the therapeutic objectives disclosed above.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Electrophoretic Mobility Shift Assays

Electrophoretic Mobility Shift Assays (EMSA) were performed to determine whether the addition of various concentrations of polyamides specific for the sequences flanking the TATA box of the HER2/neu promoter could interfere with the DNA binding activity of the TATA binding protein (TBP). Oligonucleotides corresponding to the HER2/neu TATA box and the adjacent sequences were synthesized. The first oligonucleotide, HERTATA1, has the sequence

5'-GCTGCTTGAGGAAG**TATA**AGAATGAAGTTGTGAAG-3' (the TATA box is in bold). The complementary oligonucleotide, HERTATA2, has the sequence

5'-CTTCACAACTTCATTCTTATACTTCCTCAAGCAGC-3'. These complementary 35 base oligonucleotides were 5' end-labeled with γ -³²P-ATP and T4 polynucleotide kinase and then annealed to give a double-stranded 35 base pair oligonucleotide. This oligonucleotide was then used in electrophoretic mobility shift assays employing 5% nondenaturing polyacrylamide gels (29:1 acrylamide to bisacrylamide) containing 4 mM MgCl₂ and 0.02% (v/v) NP-40 nonionic detergent along with 44 mM Tris-borate, pH 8.3, 1 mM EDTA. The labeled oligo, at a

concentration of 0.1 nM, was reacted with 1 nM final concentration of TBP (Promega) in a reaction volume of 20 μ l, containing 10% glycerol (v/v), 20 mM HEPES-OH, pH 7.9, 25 mM KCl, 0.025% NP-40 (v/v), 100 μ g/ml bovine serum albumin, 0.5 mM dithiothreitol, 0.8 mM spermidine, 0.1 mM EDTA, 2 mM $MgCl_2$.

5 Various concentrations of the different polyamides, ranging from 0.1 nM to 30 nM, were added to this binding reaction. The reactions were subjected to polyacrylamide gel electrophoresis and the dried gels were subsequently imaged and quantified using a Molecular Dynamics phosphorimager equipped with ImageQuant software. The results are shown in (Figure 4). It is clear that the HER2/neu-specific
10 polyamides (polyamides HER2-1 (Figure 4A), 70 (Figure 4B, squares), and RPR70, of composition ImPyPyPy- γ -PyPyPyPy- β -RPR, (Figure 4B, x's) significantly decrease TBP binding to the HER2/neu TATA box *in vitro*. A control polyamide 86 (Figure 4B, circles), which is not specific for the HER2/neu TATA box, has little effect on the binding of TBP to the HER2/neu TATA box.

15 "RPR" indicates the presence of a charged arginine-proline-arginine tail on the polyamide.

EXAMPLE II

Inhibition of Transcription *in vitro* In a Cell-Free System

20 The restriction endonuclease Dra I was used to linearize the plasmid pGEM/HNP, containing the HER2/neu promoter (Ebbinghaus, et al. (1993)), to produce a template for transcription. This template contained 270 base pairs of the HER2/neu promoter as well as 400 base pairs of downstream sequence. Transcription reactions were performed in 20 μ l reactions containing 100ng of template DNA and 2
25 μ l of a HeLa cell nuclear extract in a reaction volume of 25 μ l as recommended by the supplier (Promega). These reactions were incubated at 30° C for 1 hour in the presence of 10 μ Ci α - 32 P-GTP along with unlabeled nucleoside triphosphates at 0.6 mM and 20 μ M GTP. The labeled transcripts were purified using RNazol as recommended by the supplier (Teltest) and subjected to denaturing polyacrylamide
30 gel electrophoresis on 8% polyacrylamide gels containing 8.3 M urea and 88 mM Tris-borate, pH 8.3, 2 mM EDTA.

The dried gels were imaged and quantitated using a phosphorimager (Molecular Dynamics). The *in vitro* transcription from this HER2/neu promoter template was inhibited by the addition of increasing amounts of polyamide HER2-1 (Figure 5). The addition of 10 nM HER2-1 had no effect, relative to the reaction with no added polyamide, while the addition of 100nM repressed transcription two-fold. As a control, *in vitro* transcription was also performed using the unrelated cytomegalovirus (CMV) promoter as a transcription template. Addition of 10 nM or 100nM polyamide HER2-1 to this template did not significantly decrease the level of transcription.

EXAMPLE III

Inhibition of HER2/neu Expression in Cell Culture

A number of breast cancer cell lines were obtained from the American Type Culture Collection and maintained in cell culture. These cell lines include breast cancer cells in which the HER2/neu gene is amplified and greatly overexpressed (SK-BR-3 and ZR 75-1) and breast cancer cells that have normal copy numbers of the HER2/neu gene and express HER2/neu at normal low levels (Hs 578T). Various concentrations of polyamides were added directly into the appropriate cell culture medium supplemented with 10% (v/v) fetal bovine serum during the growth phase of these cells at 37° C in a 5% CO₂/air mixture. For the initial experiments, polyamide HER2-1 was added to the medium at various concentrations. A control polyamide (HIV-1) was added in various concentrations to different flasks of cells. Polyamide HIV-1 is similar in structure to HER2-1, but does not specifically recognize the HER2/neu TATA box or its adjacent sequences.

In subsequent experiments, the cell line SK-BR-3 was treated with polyamides for 6 days. In these experiments, polyamide HER2-1 and polyamide 70 in separate experiments were added to the cell culture media for a final concentration of 0.5µM. After 3 days of incubation, fresh media and fresh polyamide were added to the cells. These cells were incubated for an additional 3 days and then harvested for RNA extraction.

Once the cells had grown to confluence in 75 cm² culture flasks, the polyamide-treated breast cancer cells were harvested by treating the adherent cells with 2 ml of 0.05% trypsin-0.53 mM EDTA (Gibco BRL) to detach the cells from the culture flask. These cells were collected and pelleted in a clinical centrifuge at 5000 rpm (IEC). The cells were rinsed with cold 1x phosphate buffered saline (PBS) and pelleted in the clinical centrifuge. Total RNA was extracted from the cells using RNAzol (Teltest). To a packed cell volume of approximately 100 µl, 1ml of RNAzol and 100µl of chloroform were added. This mixture was vortexed for 10 seconds and placed on ice for 10 minutes. The mixture was then spun for 15 minutes in a microfuge at 4° C at 14,000 g. The top layer was removed and total RNA precipitated with one volume of isopropanol. The RNA pellet was washed with 70% ethanol, dried under vacuum, and resuspended in 50-100 µl of RNase-free (DEPC-treated) water containing 1µl of RNasin (40 units).

The effects of polyamide addition were subsequently analyzed using reverse transcriptase (RT)-polymerase chain reaction (PCR) as an assay for the relative level of HER2/neu mRNA. These HER2/neu mRNA levels should correlate with the amount of transcription from the HER2/neu promoter, allowing the determination of whether polyamide HER2-1 has any effect on transcription *in vivo*. Using PCR primers specific for the HER2/neu oncogene, the PCR product will correspond to HER2/neu cDNA, reflecting the relative levels of HER2/neu mRNA. The PCR primers were: (Her2A) 5'-GCTGGCCCGATGTATTTGATGGT-3' and (Her2B) 5'-GTTCTCTGCCGTAGGTGTCCCTTT-3', and 50 ng of each were used in PCR reactions as described below.

The relative amounts of HER2/neu mRNA from the various cells can be determined using reverse transcriptase (RT)-polymerase chain reaction (PCR). After total RNA has been extracted from the polyamide-treated breast cancer cells, as described above, the concentration of total RNA is determined by spectrophotometry (using the optical density at 260 nM) for each different cell type and polyamide concentration. An equal amount of total RNA (10 ng) is used for each RT-PCR. RT-PCR was carried out using the Reverse Transcription System kit (Promega). Using an oligo dT primer, cDNAs are synthesized from the mRNA templates by the enzyme

reverse transcriptase at 42° C for 25 min, as recommended. These cDNAs are then used as templates for PCR. Using the buffers and Taq polymerase provided in the kit, PCR was carried out at 26 cycles of denaturation at 94° C for 45 seconds, annealing at 60° C for 45 seconds, and extension at 72° C for 2 minutes. Five µCi of the radioactive nucleotide α -³²P-dATP is included in the PCR step to produce a radiolabeled PCR product which can be analyzed on an acrylamide gel and visualized by autoradiography. The relative amount of PCR product can be quantitated using a Phosphorimager (Molecular Dynamics). The level of HER2/neu mRNA from cells which have not been treated with polyamide are the positive control and are given a value of 1.0 and the HER2/neu mRNA levels for the polyamide-treated samples are given a value relative to the value for untreated cells.

The results of these RT-PCR assays are shown in Figure 6. Treatment of the cell lines SK-BR-3 and Hs 578-T with polyamide HER2-1 for 1-2 days resulted in slightly less than two-fold reduction in the relative levels of HER2/neu mRNA. The control polyamide HIV-1 had no apparent effect on the relative levels of HER2/neu mRNA. When SK-BR-3 cells were treated for 6 days with either polyamide HER2-1 or 70, the relative levels of mRNA decreased more significantly than for the 1-2 day treated cells. SK-BR-3 cells showed a 4-fold and 3-fold decrease in the relative levels of HER2/neu mRNA when treated with polyamide HER2-1 or 70, respectively. These results suggest that the polyamides can enter the cells and bind to their predetermined target nucleotide sequence, thereby interfering with the expression of that gene.

EXAMPLE IV

RNase protection assays

RNase protection assays (RPA) with specific riboprobes were used to monitor the levels of Her-2/neu mRNA in polyamide-treated and in control, untreated breast cancer cells maintained in culture. In order to synthesize a Her-2/neu mRNA-specific radiolabeled riboprobe, a segment of Her-2/neu mRNA coding sequence was inserted under the control of a T7 RNA polymerase promoter. Control experiments were performed to demonstrate the specificity of this riboprobe for Her-2/neu mRNA and to confirm that the signal obtained

provided a quantitatively reliable estimate of the relative levels of Her-2/neu mRNA in total RNA isolated from breast carcinoma cells. Varying the amounts of the test RNA in the RPA yielded a linear response signal as determined by phosphorimage analysis. Additionally, a series of commercially-available DNA templates was used to synthesize riboprobes to
5 monitor the effects of polyamide treatment on non-target genes. These genes include housekeeping genes (ribosomal protein L32, GAPDH), apoptosis-related genes (bcl-x, bax), stress-inducible genes (mcl1, GADD 45), an oncogene (c-fos), the tumor suppressor gene p53, and the cell-cycle regulatory protein gene p21.

Using these probes, mRNA levels for the control genes were found not to be affected
10 by the concentrations of a polyamide that inhibits Her-2/neu mRNA expression. The polyamide used in this experiment (**1b**; ImPy- β -PyIm-D-PyPyPyPyPy- β -Dp) was designed to bind the sequence immediately adjacent to and overlapping the Her-2/neu TATA element (see Figure 7, above) and exhibits an apparent dissociation constant of 0.26 nM as determined by DNase I footprint analysis. Cells were incubated with varying concentrations of
15 polyamide for 72 hours and total cellular RNA was then isolated. Equivalent amounts of RNA from control and treated cells were then used for riboprobe protection assays. Polyamide **1b** was found to be an effective inhibitor of Her-2/neu mRNA synthesis in the breast carcinoma cell line SK-BR-3, in which the Her-2/neu gene is overexpressed on the order of 50-fold relative to normal breast tissue. At a polyamide **1b** concentration of 0.3 μ M,
20 approximately 70% inhibition of transcription was observed. Higher concentrations of polyamide have been observed to yield higher levels of inhibition (not shown).

The lack of inhibition of control gene mRNA synthesis is a strong indication that polyamide treatment of the SK-BR-3 breast cancer cells does not induce apoptosis or a stress-related response. Moreover, polyamide treatment does not influence cell growth or density,
25 and no cytotoxicity has been observed. In control experiments, fraction of live cells in the presence or absence of polyamide treatment was determined by trypan blue staining. The treatment of the breast cancer cell line SK-BR-3 with polyamide **1b** up to a final concentration of 3 μ M in the culture medium was found to have no effect on cell viability.

EXAMPLE V

Polyamide stability

The uptake and stability of a radiolabeled pyrrole-imidazole polyamide was monitored in breast cancer cells maintained in culture. The polyamide used in this experiment was an eight ring compound (ImPyPyPy-D-PyPyPyPy- β -CT₂OH, where CT₂OH is tritiatol), similar in structure to the polyamides used in transcription experiments, with the exception that a tritiatol group was incorporated at the terminus in place of Dp. These experiments have been conducted with two different breast carcinoma cell lines (MCF-7 and ZR-75-1) and qualitatively similar results were obtained. Concentrations of polyamide in nuclear and cytoplasmic compartments of the cells were estimated after incubation with varying concentrations of tritiated polyamide for a fixed time of 72 hours. These data were obtained under the same protocol as used for the RPA experiment described above. The results demonstrate the abundant concentration of polyamide in the cell nucleus in a concentration dependent fashion. In a separate analysis, a time course of polyamide import into ZR-75-1 and MCF-7 cells was performed. In this experiment, the cytoplasmic fraction was further fractionated into cytosol and membranes and the nuclear fraction was fractionated into soluble nucleoplasm and insoluble chromatin, with the expected result that the polyamide is highly enriched in chromatin.

The stability of the tritiated polyamide in cells after incubation in culture medium without the compound was also monitored. In this experiment, cells were first incubated in culture medium containing 0.1 μ M radiolabeled polyamide for 24 hours. After this incubation, the culture medium was removed and fresh medium lacking polyamide was added. Serum concentration was reduced to 0.1% to prevent cell proliferation during subsequent incubation in the absence of polyamide. Cells were harvested at 24 hour intervals for up to six days. We find that the amount of cytoplasmic polyamide declines by half after 24 hours, whereas the chromatin-bound polyamide appears to be more stable. Similar results were obtained with ZR-75-1 cells. Additional experiments will determine whether there is any change in the chemical composition of the polyamide during cell treatment, such as degradation or incorporation of the radioisotope into other molecules. Taken together these results suggest that the pyrrole-imidazole polyamides are cell permeable and are concentrated in the nucleus, and bound to chromatin. The high levels of polyamides found in the chromatin indicate that the compounds bind to multiple sites in the human genome, as would be expected given the short polyamide target sequences (which would be present millions of

times in the genome). The absence of cytotoxicity suggests that although multiple sites are occupied by the polyamides, deleterious non-specific effects on genome-wide transcription are absent. Nonetheless, future studies will be conducted with polyamides designed to target more complex DNA sequences which should be promoter-specific, as described in the application.

EXAMPLE VI

Cell Motility Assays

In collaboration with the laboratory of Dr. Vito Quaranta (of the Department of Cell Biology, The Scripps Research Institute), the effect of polyamide treatment on motility of MCF-7 breast carcinoma cells *in vitro* was monitored. This assay monitors the attachment of cells to a protein-coated membrane and subsequent migration of the cells through the membrane. The ability of cells to transit through the membrane is taken as an indication of the metastatic potential of the cells. In control experiments it was determined that polyamide treatment did not affect the ability of the cells to attach to membranes coated with either collagen IV, laminin-5, or fibronectin. When MCF-7 cells were treated with polyamide Her2-1 or 70 in separate experiments, approximately 50% reduction in steady-state Her-2/neu mRNA was observed after three days in culture. This inhibitory value is consistent with our results for three-day treatments of other breast carcinoma cell lines with these polyamides. As before, a control mismatch polyamide had no effect on Her-2/neu mRNA levels. Migration of the cells across the membrane was also inhibited by 50% by treatment with polyamides Her2-1 or Her2-A (at 0.5 μ M in the culture medium), but polyamide 86 was without effect on cell migration at a similar concentration. These findings were obtained with membranes coated with collagen IV, laminin-5 and fibronectin. These preliminary experiments suggest that down-regulation of Her-2/neu mRNA, and hence protein, may decrease the motility of MCF-7 cells; however, a direct link between loss of motility and Her-2/neu protein levels must be obtained in future experiments.

REFERENCES

1. Trauger, J.W., Baird, E.E., and Dervan, P.B. (1996) Subnanomolar sequence-specific recognition in the minor groove of DNA by designed ligands. *Nature* 382, 559-561.
2. Turner, J.M., Swalley, S.E., Baird, E.E., and Dervan, P.B. (1998) Aliphatic/aromatic amino acid pairings for polyamide recognition in the minor groove of DNA. *J. Am. Chem. Soc.* 120, 6219-6226.
3. White, S., Szewczyk, J.W., Turner, J.M., Baird, E.E., and Dervan, P.B. (1998) Recognition of the four Watson-Crick base pairs in the DNA minor groove by synthetic ligands. *Nature* 391, 468-471.
4. Geierstanger, B.H., Mrksich, M., Dervan, P.B., and Wemmer, D.E. (1994) Design of a G·C-specific DNA minor groove-binding peptide. *Science* 266, 646-650.
5. Kielkopf, C.L., Baird, E.E., Dervan, P.B., and Rees, D.C. (1998) Structural basis for GC recognition in the DNA minor groove. *Nature Struct. Biol.* 5, 104-109.
6. Kielkopf, C.L., White, S., Szewczyk, J.W., Turner, J.M., Baird, E.E., Dervan, P.B., and Rees, D.C. (1998) A structural basis for recognition of A·T and T·A base pairs in the minor groove of B-DNA. *Science* 282, 111-115.
7. Baird, E.E., and Dervan, P.B. (1996) Solid phase synthesis of polyamides containing imidazole and pyrrole amino acids. *J. Am. Chem. Soc.* 118, 6141-6146.
8. Chang, C.H., *et al.* (1997) ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene* 14, 1617-1622.
9. Ebbinghaus, S.W., Gee, J.E., Rodu, B., Mayfield, C.A., Sanders, G., and Miller, D.M. (1993) Triplex formation inhibits HER-2/neu transcription in vitro. *J. Clin. Invest.* 92, 2433-2439.
10. Mizuguchi, G., Kanei-Ishii, C., Sawazaki, T., Horikoshi, M., Roeder, R.G., Yamamoto, T., and Ishii, S. (1994) Independent control of transcription initiations from two sites by an initiator-like element and TATA box in the human c-erbB-2 promoter. *FEBS Lett.* 348, 80-88.

All references cited herein are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

5 While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice
10 within the art to which the invention pertains and as may be applied to the essential features herein before set forth as follows in the scope of the appended claims.